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EXHIBIT 3

Human duodenal enteroendocrine cells: source of both incretin peptides, GLP-1 and GIP

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Theodorakis, Michael J., Olga Carlson, Spyros Michopoulos, Máire E. Doyle, Magdalena Juhaszova, Kalliopi Petraki, and Josephine M. Egan. Human duodenal enteroendocrine cells: source of both incretin peptides, GLP-1 and GIP. *Am J Physiol Endocrinol Metab* 290: E550–E559, 2006. First published October 4, 2005; doi:10.1152/ajpendo.00326.2004.—Among the products of enteroendocrine cells are the incretins glucagon-like peptide-1 (GLP-1, secreted by L cells) and glucose-dependent insulinotropic peptide (GIP, secreted by K cells). These are key modulators of insulin secretion, glucose homeostasis, and gastric emptying. Because of the rapid early rise of GLP-1 in plasma after oral glucose, we wished to definitively establish the absence or presence of L cells, as well as the relative distribution of the incretin cell types in human duodenum. We confirmed the presence of proglucagon and pro-GIP genes, their products, and glucosensory molecules by tissue immunohistochemistry and RT-PCR of laser-captured, single duodenal cells. We also assayed plasma glucose, incretin, and insulin levels in subjects with normal glucose tolerance and type 2 diabetes for 120 min after they ingested 75 g of glucose. Subjects with normal glucose tolerance ($n = 14$) had as many L cells (15 ± 1), expressed per 1,000 gut epithelial cells, as K cells (13 ± 1), with some containing both hormones (L/K cells, 5 ± 1). In type 2 diabetes, the number of L and L/K cells was increased (26 ± 2; $P < 0.001$ and 9 ± 1; $P < 0.001$, respectively). Both L and K cells contained glucokinase and glucose transporter-1, -2, and -3. Newly diagnosed type 2 diabetic subjects had increased plasma GLP-1 levels between 20 and 80 min, concurrently with rising plasma insulin levels. Significant coexpression of the main incretin peptides occurs in human duodenum. L and K cells are present in equal numbers. New onset type 2 diabetes is associated with a shift to the L phenotype.

duodenum; euglycemia; type 2 diabetes; glucagon-like peptide-1; gastric inhibitory polypeptide

THE GASTRO-ENTERO-PANCREATIC SYSTEM, within which pancreatic islets of Langerhans lie, is the largest system of endocrine cells in the body in terms of both number of cells and variety of hormones produced (52). Also within this system lie the incretin-producing cells of the gut, whose products constitute key mediators of food-stimulated, glucose-dependent insulin secretion, accounting for up to 60% of the insulin secretory response after an oral glucose load (44). Glucose-dependent insulinotropic peptide [GIP (also known as gastric inhibitory polypeptide)] and glucagon-like peptide-1 (GLP-1) are the two known incretins (40, 61). The pro-GIP gene is expressed in K cells, the majority of which are located in duodenum and upper jejunum (39). The proglucagon gene is expressed in α-cells of islets of Langerhans, L cells of the gut, and specialized neurons; most L cells are thought to reside in lower jejunum and terminal ileum. (20, 22). Posttranslational processing of proglucagon in α-cells generates glucagon, but in L cells GLP-1 and GLP-2 are the products with known functions (13, 15). In humans, GLP-1 exists mainly as a COOH-terminally amidated form, GLP-1-(7–36) amide, and a minor glycine extended form, GLP-1-(7–37) (49); both are insulinotropic. Tissue and plasma dipeptidyl peptidase IV (DPP IV) rapidly inactivates both GIP and GLP-1 as insulinotropic factors by cleaving their first two NH_2-terminal amino acids (13, 15).

Despite the importance of incretins, not only for insulin secretion but for food intake, gastric emptying, and acid secretion, energy expenditure, and hormonal regulation, especially in the postprandial state, knowledge of their cells of origin in the gastrointestinal mucosa is scarce. It has long been described as a paradox that the apparent rapid increase in plasma levels of GLP-1 (21) could not be readily attributed to the presence of a distinct cell population in the proximal intestine (20, 22), as is the case for GIP (20), despite the fact that it has been well established for almost three decades that enteroglucagon was expressed in human duodenum, albeit at ~20-fold lower density than in the human ileum, although the opposite gradient was true for GIP (3, 26).

We examined duodenal biopsy tissue from 31 subjects with euglycemia and type 2 diabetes (T2DM) taken during routine endoscopy. In parallel, we determined plasma responses of circulating intact and total GLP-1, as well as GIP and insulin, to a 2-h oral glucose tolerance test (OGTT), with subsequent frequent blood sampling.

MATERIALS AND METHODS

Study design. We screened Baltimore Longitudinal Study on Aging (BLSA) participants who were not on prescribed glucose-lowering medications and had oral glucose tolerance testing at their most recent visit. From that, we recruited all 17 subjects with newly diagnosed T2DM (by both fasting plasma glucose ≥126 mg/dl and 2-h plasma glucose ≥200 mg/dl) and 36 subjects with normal fasting and 2-h glucose levels to undergo a modified OGTT. In addition, we examined duodenal tissue from 31 non-BLSA subjects: 14 with fasting euglycemia and no clinical suspicion of T2DM, based on medical and family history and clinical and laboratory examination, and 17 with diagnosis of T2DM based on and confirmed by fasting plasma glucose (by physicians' and the subjects' personal communications they had

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not been receiving glucose-lowering drugs). They were screened and selected from a pool of volunteer subjects who underwent upper endoscopy for clinical protocols unrelated to duodenal pathologies (further confirmed by histological evaluation). Subjects whose duodenal specimens showed any abnormalities in histopathology or had a positive test for *Helicobacter pylori* were excluded from the study. Standard anesthesia and endoscopy methodologies were followed in all cases after fasting for a minimum of 4 h. Two separate mucosal specimens were obtained from the first and second portions of the duodenum. None of the participants who underwent upper endoscopy were taking any medication during at least a 3-mo time period before the study; they were nonsmokers and had no concurrent disease. All subjects in this study had no statistical differences in their age and body mass index. Characteristics of both study populations are shown in Table 1. The Committees on Human Investigation at each investigator's institution approved the studies. All volunteers provided written informed consent in accordance with the Helsinki II declaration.

Modified OGTT in BLSA subjects. After an overnight fast, participants drank 75 g of glucose (SunDex; Fisherbrand, Pittsburgh, PA). Blood samples were collected on ice into EDTA-coated tubes (1.5 μg/ml blood) containing aprotinin (40 μl/ml blood; Trasylol, Serological Proteins, Kankakee, IL) and a DPP IV inhibitor (no. DPP4; 10 μl/ml blood, Linco Research) at baseline (*time 0*), then at 5, 10, 15, 20, 40, 60, 80, 100, and 120 min after glucose ingestion for measurements of plasma glucose, insulin, C-peptide, glucagon, total and intact (NH_2-terminal) GLP-1, and total and intact (NH_2-terminal) GIP levels. To assess early-phase insulin secretion, we used the insulinogenic index (7), calculated as the ratio of the increment in the plasma insulin level to that in the plasma glucose level during the first 20 min after ingestion of glucose; the lower the index, the worse the insulin secretion. We also determined insulin resistance by the homeostatic model assessment (HOMA-IR) (37), calculated as the product of fasting insulin (in μU/ml) and fasting glucose (in mmol/l) divided by 22.5. Lower insulin resistance values indicate better insulin sensitivity. We calculated the area under the curve (AUC) for plasma insulin and incretin concentrations vs. time by the trapezoidal method (33). Body mass index was calculated as body weight (in kg) divided by the square of the height (in m), measured manually by a medical scale (Seca, Hanover, MD).

Plasma hormone and biochemical assays. We assayed plasma samples for insulin and C-peptide by ELISA (ALPCO Diagnostics, Windham, NH), with a detection limit of 1 μU/ml and 20 pmol, respectively. Cross-reactivity of the insulin antibody for C-peptide and vice versa is <0.1%. We measured plasma glucagon by radioimmunoassay (Linco Research, St. Charles, MO), with a detection limit of 2 pg (100 μl plasma), and intact GLP-1 [-(7–36) amide and -(7–37)] and total GLP-1 [-(7–36) amide, -(7–37), -(9–37), and -(9–36) amide] by ELISA and radioimmunoassay, respectively (both from Linco Research). We employed an NH_2-terminally directed ELISA assay for plasma intact GIP determinations (Peninsula Laboratories, San Carlos, CA), because reports (12) suggest that such an approach yields optimal accuracy, with a detection level of 0.1 pmol (50 μl plasma) as well as a radioimmunoassay for total plasma GIP determinations (Phoenix Laboratories, Belmont, CA), with a detection level of 4 pmol (100 μl plasma), as previously described (57) [see APPENDIX for GIP-(1–42) and GIP-(3–42) by ELISA and GIP-(1–42)

Table 1. *Clinical, demographic characteristics, and fasting serum biochemical values of the subjects*

		BLSA OGTT Group		Endoscopy Group	
Variable	Quantity	Normal glucose tolerance (n = 36)	Type 2 diabetes (n = 17)	Fasting euglycemia (n = 14)	Type 2 diabetes (n = 17)
Age	yr	71±14	71±10	62±14	65±10
Range		39–93	54–87	47–79	44–77
Race	%				
Caucasian		78	65	100	100
African American		19	29		
Native American		3	6		
Sex					
Female		16	3	4	5
Male		20	14	10	12
Hb A_{1c}	%	5.32±0.08	6.96±0.20†	4.91±0.20	6.28±0.10†
BMI	kg/m^2	27±5	30±4	24±3	29±3
Range		19–39	23–40	20–35	24–38
Fasting plasma glucose	mmol/l	5.1±0.6	8.2±0.2†	4.84±0.2	7.54±0.3†
Fasting plasma insulin	pmol/l	36±6	66±6†		
Plasma glucagon	ng/l	59±9	107±12†		
Fasting plasma GLP-1 (intact)	pmol/l	5±1	5±1		
Fasting plasma GLP-1 (total)	pmol/l	6±1	12±2†		
Fasting plasma GIP (intact)	pmol/l	31±3	30±2		
Fasting plasma GIP (total)	pmol/l	32±3	45±4		
Insulin AUC (0–20/20–120 min)	$pmol \cdot l^{-1} \cdot min$	2,790±300/25,794±2,892*	1,800±222/22,878±5,400		
Intact GLP-1 AUC (0–20/20–120 min)	$pmol \cdot l^{-1} \cdot min$	179±16/742±55	183±12/987±79*		
Total GLP-1 AUC (0–20/20–120 min)	$pmol \cdot l^{-1} \cdot min$	322±50/1,549±196	423±43/2,443±217*		
Intact GIP AUC (0–20/20–120 min)	$pmol \cdot l^{-1} \cdot min$	1,537±207/9,492±548	1,364±122/7,731±674		
Total GIP AUC (0–20/20–120 min)	$pmol \cdot l^{-1} \cdot min$	2,392±257/13,894±623	2,586±196/15,539±732		
HOMA-IR		1.46±0.17	3.80±0.43†		
Insulinogenic index		0.87±0.08	0.34±0.11†		
Total cholesterol	mmol/l (mg/dl)	4.97±0.18 (192±7)	4.76±0.21 (184±8)		
HDL cholesterol	mmol/l (mg/dl)	1.5±0 (60±3)	1.1±0** (43±2†)		
LDL cholesterol	mmol/l (mg/dl)	2.94±0.15 (115±6)	2.56±0.18 (100±7)		
Triglycerides	mmol/l (mg/dl)	1±0 (83±6)	2±0** (204±35†)		

*Statistically significant difference compared to subjects with normal glucose tolerance ($P < 0.05$); †statistically significant difference compared to subjects with normal glucose tolerance ($P < 0.001$). **Values are means ± SE. BLSA, Baltimore Longitudinal Study on Aging; OGTT, oral glucose tolerance test; Hb A_{1c}, glycosylated hemoglobin; BMI, body mass index; GLP-1, glucagon-like peptide-1; GIP, glucose-dependent insulinotropic peptide; AUC, area under the curve; HOMA-IR, insulin resistance by homeostatic model assessment; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

by RIA]. The assay for intact GLP-1 has no cross-reactivity for GLP-1-(9–36) amide or -(9–37), GLP-2, or glucagon and has a detection limit of 2 pmol (100 μl plasma). The radioimmunoassay for total GLP-1 has <0.01% and 0.2% cross-reactivity for GLP-2 and glucagon, respectively, and has a detection limit of 3 pmol (300 μl plasma). The intra-assay variation coefficients for intact GLP-1, total GLP-1, intact GIP, total GIP, insulin, C-peptide, and glucagon were 7, 13, 5, 12, 3.6, 3.6, and 4.8%, respectively, and the interassay variation coefficients for intact GLP-1, total GLP-1, intact GIP, insulin, C-peptide, and glucagon were 8, 17, 14, 2.5, 3.3, and 12%, respectively. We have found that freezing causes a reduction in intact GIP and GLP-1 absolute values by ~50% compared with fresh plasma. Hence, these were measured on the day the plasma was separated. Total GIP levels were all assayed at the same time in aliquots frozen just once. Hence, there was no interassay variation. We performed all assays in duplicate, in low-absorbency tubes, also suitable for RNA storage (cat. no. 3443; Neptune Plastics, San Diego, CA). We measured plasma glucose levels with a glucose analyzer (Beckman Instruments, Brea, CA) and glycosylated hemoglobin ($Hb\ A_{1c}$) with an automated Diastat analyzer (Bio-Rad Laboratories, Hercules, CA). Plasma lipid levels were determined by the Clinical Core Laboratory Unit (National Institute on Aging/National Institutes of Health, Baltimore, MD) using an AutoAnalyzer (Synchron CX-5 Delta, Beckman Instruments).

Tissue processing and immunofluorescence. Biopsy specimens (10–15 μg) from both portions of duodena from each subject were fixed immediately in 4% phosphate-buffered paraformaldehyde (PBS) and embedded in paraffin, and 7-μm-thick sections were mounted on charged glass slides and processed using standard methodologics. Primary antibodies used were biotinylated and nonbiotinylated: rabbit anti-GLP-1-(7–37) (1:200) and rabbit anti-GLP-2 (1:200), from Phoenix Pharmaceuticals; rabbit anti-GLP-1-(7–36) amide (1:200), from Peninsula Laboratories; goat anti-glucokinase (1:50), goat anti-GLUT1 (1:80), goat anti-GLUT2 (1:100), and goat anti-GLUT3 (1:150), from Santa Cruz Biotechnology (Santa Cruz, CA); and a highly specific rabbit anti-human GLUT3 (1:500; gift from Ian Simpson, Pennsylvania State College of Medicine, Hershey, PA). Because many anti-GIP antibodies cross-react with antigens in human islet α-cells (31, 56), we used a specific rabbit anti-GIP antibody (1:500; gift from Dariush Elahi, Massachusetts General Hospital, Harvard University, Boston, MA) that exhibited no such immunoreactivity, as we confirmed. Specificity of the anti-GLP-1 and anti-GIP antibodies was ensured by preabsorbing with GIP, GLP-1, and glucagon (the same approach was employed to ensure specificity of the goat antisera bodies). Negative controls were substitution of primary antiserum with (biotinylated or nonbiotinylated) rabbit immunoglobulins and goat serum (DAKO, Carpinteria, CA) or antibody diluent alone. Fluorescent antibodies used were Alexa fluor 488, 568, and 633, and donkey anti-rabbit or donkey anti-goat immunoglobulins (1:1,000; Molecular Probes, Eugene, OR). When examining coexpression of GIP and GLP-1 or GIP and GLP-2, we used biotinylated rabbit anti-GLP-1-(7–37) and anti-GLP-2, and secondary labels were Alexa fluor 488 and 633 streptavidin conjugates, respectively, 1:200. For coexpression of GLUT3 and GIP, we used the goat anti-GLUT3 antibody. We performed double labeling of the sections as previously described (9), according to the procedure of Jackson Immunoresearch. Under those conditions, because all of the primary antibodies were raised in rabbit, we performed control experiments where the second primary antisera were omitted to verify that the second labeled secondary antisera (anti-rabbit fluorophore) was not binding to the first primary antisera. To control for the specificity of all antibodies, their immunoreactivity was also blocked by preincubation with the corresponding antigenic peptides. To verify this further, we also conducted double-labeling experiments, as described above, by the use of two mouse monoclonal antibodies specifically for the amidated COOH terminus of the (1–36) and (7–36) peptide, respectively (1:150; Statens Serum Institut, Copenhagen, Denmark), and an additional mouse monoclonal anti-human GLP-1-(7–36) amide antibody (1:80; Immundiagnostik, Bensheim, Germany; data not shown), an approach that confirmed the results of the double-labeling experiments. Immunofluorescence with all the antibodies employed in gut tissue staining was also used on sectioned human pancreatic islets that served as controls for antibody specificity. Immunofluorescence was visualized with a Zeiss LSM-410 inverted laser confocal microsope (Carl Zeiss, Oberkochen, Germany). Laser and filter settings were compatible and selective for applied fluorophores that were excited with the 488-nm line of a krypton-argon laser and fluorescence emission collected at 515–565 nm, the 633-nm helium-neon laser and recorded in the prescnce of a 670–810-nm band-pass cmission filter, or the 568-nm laser linc and emission collected at 590–610 nm. Images of five random fields were analyzed in each section. For these, we used a Zeiss 63×/1.4 numerical aperture oil immersion objective, and the confocal pinhole was set to obtain spatial resolution of 0.4 μm in the horizontal plane and 1 μm in the axial dimension. MetaMorph 4.6.3 software (Universal Imaging, West Chester, PA) was used for image processing and morphometric analysis. Immunostained cells and nuclei were counted after color separation and image thresholding, both manually and by software validation.

Laser capture microdissection. We used the PixCell II Workstation (Arcturus Engineering, Mountain View, CA) to perform laser capture microdissection and image acquisition. Briefly, single staining for GLP-1 or GIP was performed on separate slide preparations labeled with the Alexa fluor 488 secondary antibody. After the final washing steps, having always been protected from light, slides were dehydrated, cleared in xylene, air dried, and immediately processed for microdissection. Laser power was adjusted to 30–55 mW, and pulse duration of 6–8 ms was utilized to melt a circular area (spot size, 7.5 μm in diameter) on the polymer surface of the 4-μm adherence rail cap to capture single cells. Successful acquisition of target cells was constantly monitored by a color camera and high-resolution monitor (Sony, Tokyo, Japan). Single-cell capture was confirmed by inverted light microscopy of the collected cell material on the thermoplastic film lining the cap surface and documented by careful analysis of the camera-acquired images. Also, nonstained enterocytes were always captured, serving as a negative control for incretin expression. Caps were inserted into 500-μl tubes, and adhered cells were promptly forwarded to reverse transcription.

RT-PCR. Downstream processing of the captured pooled cells was done by means of the One-Step reverse transcription kit (Qiagen, Valencia, CA). After lysis in 25 μl of buffer, release of RNA and inactivation of RNAases was achieved by 10 min of incubation at 75°C, followed by the addition of DNase I (concentration <0.04 U/μl) for genomic DNA degradation at 37°C for 15 min and then DNase inactivation at 75°C for 5 min. One-step RT-PCR was performed according to the supplied protocol in a reaction volume of 50 μl in a Peltier thermal cycler (PTC-225 DNA Engine Tetrad Cycler; MJ Research, Waltham, MA), using published human gene-specific primer sequences (Integrated DNA Technologies, Coralville, IA) as follows (5′-3′ forward/reverse primers, respectively): proglucagon, GTAATGCTGGTACAAGGCAG/TTATAAAGTCCCTGGCGGCA; pro-GIP, CGAAGACCTTTGCTCTGCTCTGCTGCTGCT/ACCT-GAGCCTGCAGAGGTTTGTCTG. Reverse transcription was performed at 50°C for 30 min. After an initial activation step at 95°C for 15 min, the PCR conditions were as follows (in all instances, the cycles were followed by a final extension step at 72°C for 10 min): for proglucagon, denaturing at 94°C/1 min, annealing at 55°C/1 min, and extension at 72°C/1 min for 35 cycles; for pro-GIP, denaturing at 94°C/1 min, annealing at 60°C/1 min, and extension at 72°C/1 min for 35 cycles. In each procedure, two negative controls were included, where water was substituted for the omitted reverse transcriptase or template. RNA was isolated, as previously described (19), from human pancreatic islets provided by Dr. David M. Harlan (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD), which served as a positive proglucagon (and negative pro-GIP)

control, as well as from whole human duodenal biopsy tissue, which served as positive control for pro-GIP. PCR products were resolved in 2% agarose gel with ethidium bromide and visualized under UV light.

Statistical analysis. All values are expressed as means ± SE, and all data were analyzed using SAS 8.2 software (SAS Institute, Cary, NC). Standard methods were used to compute means and SE. We tested all values for normality of distribution by the Kolmogorov-Smirnov test. We compared mean values of plasma glucose and hormone levels by repeated-measures ANOVA and Bonferroni's multiple comparison post hoc test and mean values of HOMA-IR, insulinogenic index, age, body mass index, Hb A_{1c}, lipids, and AUC in plasma hormone levels between groups by an unpaired *t*-test. We used the paired *t*-test to compare mean values of plasma insulin at 20 and 60 min in subjects with T2DM. We applied nonparametric statistics to compare mean numbers of counted cells that exhibited immunoreactivity for GLP-1, GIP, or the two hormones together between subject groups. Because cell count data had a skewed distribution, values were also log transformed for analysis, but for clarity of interpretation, results are expressed as untransformed values. *P* values of <0.05 were regarded as indicating statistical significance.

RESULTS

Diabetic subjects had a metabolic profile typical for this disease (Table 1): significantly higher insulin resistance indexes, Hb A_{1c}, and triglyceride levels, coupled with lower HDL cholesterol levels compared with subjects with normal glucose tolerance.

Subjects with T2DM had increased fasting plasma insulin levels and a significantly reduced insulinogenic index (represented by significantly lower AUC for insulin) from 0 to 20 min after oral glucose compared with subjects with normal glucose tolerance (Fig. 1 and Table 1). Notably, between 20 and 60 min after glucose, there was a significant increase in plasma insulin levels in T2DM ($P < 0.01$), which corresponded to the peak plasma levels of GLP-1 in this group. As shown previously (10), fasting glucagon levels were significantly higher in T2DM (Table 1); at 2 h, levels remained almost twofold higher compared with the normal glucose-tolerant state (Fig. 1).

Fasting plasma levels of intact GLP-1 and its levels from 0 to 20 min after glucose were similar in the two groups (Fig. 1 and Table 1). Both groups exhibited a similar rise in intact GLP-1 levels in plasma during the first 20 min that peaked at 15 min in nondiabetic subjects but continued rising in those with T2DM. From 20 to 120 min, however, the AUC was significantly greater in T2DM compared with subjects that had normal glucose tolerance (Fig. 1 and Table 1), because intact GLP-1 plasma levels were significantly higher in T2DM after the first 20 min and up to 80 min compared with the nondiabetic subjects.

Fasting plasma total GLP-1 levels were significantly higher ($P < 0.001$) in subjects with T2DM (12 ± 2 pmol/l) compared with normal glucose tolerance (6 ± 1 pmol/l; Fig. 1 and Table 1). Similar to intact GLP-1, the AUC for total GLP-1 from 20 to 120 min was significantly greater in T2DM compared with subjects with normal glucose tolerance (Table 1).

Fasting plasma intact and total GIP levels, GIP secretion pattern, and the AUC estimates after oral glucose were similar in subjects with T2DM and normal glucose tolerance (Fig. 1 and Table 1).

In all of the 31 subjects that were biopsied, L cells were present in both portions of the duodenum (Fig. 2*A*), as determined by the presence (using multiple antibodies) of three proglucagon products: GLP-1-(7–37), GLP-1-(7–36) amide, and GLP-2 (Fig. 2, *A*, *B* and *C*, respectively). As expected (39, 61), K cells were also present (Fig. 2*D*). Some enteroendocrine cells contained both GLP-1 and GIP (Fig. 2, *F–H*). To confirm colocalization of proglucagon products with GIP, we looked for colocalization of GLP-2 with GIP (Fig. 2, *I–K*). Cells containing GLP-1 always costained for GLP-2 (data not shown). Morphologically, the cells were comet-like in shape, with a longer apical portion projecting toward the intestinal lumen and a wider basal surface toward the submucosa.

Pertinent to glucose sensing, all immunopositive incretin cells expressed glucokinase (Fig. 2, *L–N*; not shown here for K cells), and GLUT1 and GLUT2 (immunostaining data not shown), similar to β-cells. Unlike β-cells but similar to neurons (35), GLUT3 was also present in all immunopositive incretin cells (Fig. 2, *O–T*). It exhibited a granular pattern (Fig. 2*E*), unlike plasma membrane localization of neuronal GLUT3 (Fig. 2*E*, *inset*).

Cell counts were normalized per thousand propidium iodide-stained total intestinal cell nuclei. When so expressed (Fig. 3) in subjects with fasting euglycemia, the total number of L cells (15 ± 1) in both duodenal portions was similar to that of K cells (13 ± 1). However, in the diabetic population, the number of L cells was significantly higher (26 ± 2, $P < 0.001$), whereas the number of K cells (16 ± 1) was comparable to that of subjects with fasting euglycemia. In T2DM, the number of cells that contained both hormones was significantly higher (9 ± 1, $P < 0.001$) than that of subjects with fasting euglycemia (5 ± 1).

By RT-PCR (Fig. 4), we confirmed expression of the proglucagon gene, as well as the pro-GIP gene in laser-captured enteroendocrine cells from human duodenum. Cells immunolabeled for GLP-1 or GIP yielded positive PCR products when examined not only for expression of the marker peptide gene but also for the other two genes.

DISCUSSION

Our study provides new findings of significance to the pathophysiology of diabetes and other metabolic disorders. The possibility rises that L cells present in human duodenum in roughly equal numbers with K cells respond to direct nutrient contact and release GLP-1 in a manner that is analogous to secretion of GIP by the K cells. Colocalization of the two main insulinotropic peptides in the duodenum does occur, which is in accord with similar findings (41, 42) that some incretin cells in jejunum contained both GIP and GLP-1, and its extent increases significantly in the diabetic state. Contrary to reports from subjects with long-standing T2DM (47, 59), in newly diagnosed subjects late-phase GLP-1 release was augmented, concomitant with rising plasma insulin levels.

There is one report suggesting the presence of an insulinotropic peptide that is different from GIP in the porcine duodenum (55), as well as one study (41), subsequently followed up by a more extensive report (42), where the investigators described the frequency of incretin and peptide YY cell colocalization in eight sections (100 cm apart), from duodenum to caecum in swine (41). In that report, detectable GLP-1 expres-

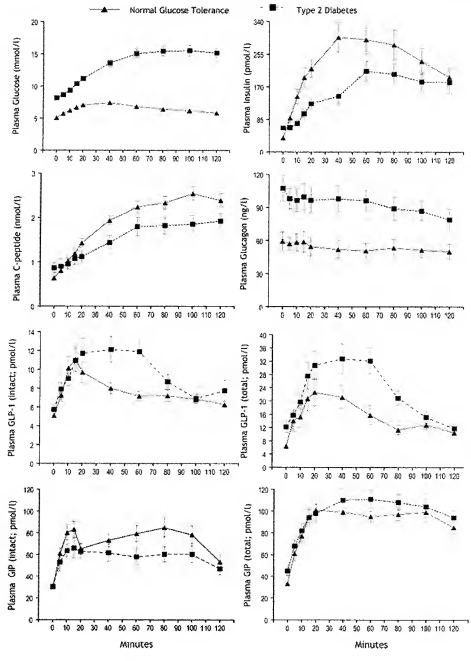

Fig. 1. Mean (±SE) plasma glucose, insulin, C-peptide, glucagon, intact (NH_2-terminal) glucagon-like peptide-1 (GLP-1), total GLP-1, intact (NH_2-terminal) glucose-dependent insulinotropic peptide (GIP), and total GIP responses during oral glucose tolerance test in our study population. Glucose (75 g) was administered at *time 0*.

sion was found (as a percentage of immunoreactive cells, with no actual images) at a distance of 0 cm from the porcine duodenum (but not the organ itself), and it is also stated that human biopsy material taken from the small intestines of three subjects (it was unclear whether duodenum was also among them) exhibited a similar pattern, but no quantification data were demonstrated in humans. In their subsequent report, the same group (42) investigated biopsies of histologically normal human small intestine obtained during surgery on the gastrointestinal tract from nine subjects (three jejunal, two midgut, and four ileal biopsies) operated for either Crohn's disease ($n = 3$), intestinal carcinoid ($n = 3$), colonic polyps ($n = 1$), cancer in cecum ($n = 1$), or cancer in papilla vateri ($n = 1$), but they did not furnish cell quantification data in humans.

On the basis of immunostaining of samples from five subjects with Crohn's disease and colon cancer, it was previously determined that L cells were abundant in distal small intestine and colon, with no L cells present in duodena (20), which implies that the rapid increase in GLP-1 plasma levels after oral glucose could not be attributed to the presence of a distinct L cell population in the proximal intestine, as is the case for K cells. However, evidence from elegant studies in dogs (11) has suggested that L cells are present in significant numbers in the duodenum and upper

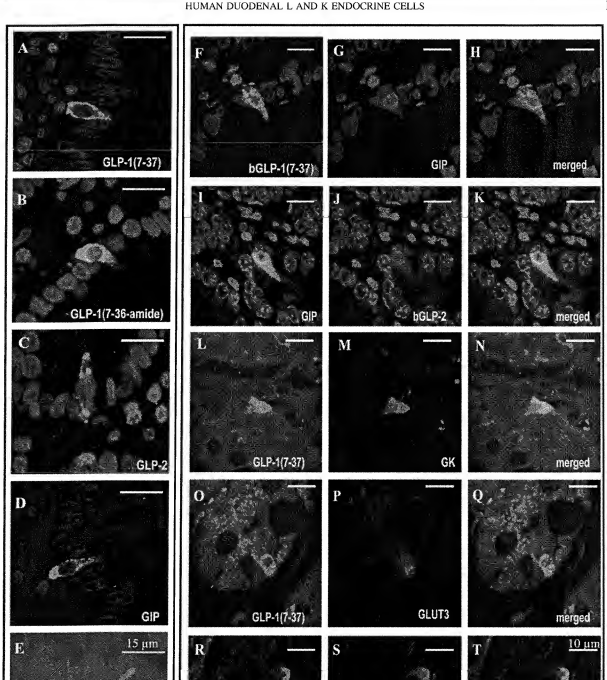

Fig. 2. Laser scanning confocal microscopy of incretin-containing cells in human duodenum. Immunofluorescent L cells containing GLP-1-(7–37), GLP-1-(7–36) amide, and GLP-2 and a K cell containing GIP are shown in green (*A*, *B*, *C*, and *D*, respectively). A duodenal cell stained with anti-human GLUT3 antibody is shown in *E* (*E*, *inset* depicts neuronal expression of GLUT3 in enteric plexus close to villi of human duodenum). A duodenal cell shows GLP-1-(7–37) (*F*, green) and GIP (*G*, blue); *H*: merged image. Similarly, a duodenal cell shows GIP (*I*, green) and GLP-2 (*J*, blue); *K*: merged image. GLP-1-(7–37)-containing cells (*L* and *O*, green) that coexpress glucokinase (*M*, red) and GLUT3 (*P*, red) are shown merged (*N* and *Q*, respectively). GIP-containing cells (*R*, green) also express GLUT3 (*S*, red); *T*: merged image. Colors represent the following: yellow (*N*, *Q*, and *T*), areas of colocalization; green, fluorescence from Alexa fluor 488; cytosolic and nuclear red staining, Alexa fluor 568 and propidium iodide, respectively; blue, Alexa fluor 633. Representative 15-μm scale bar is shown in *A–E*. Representative 10-μm scale bar is shown in *F–T* (b in *F* and *J*, refers to biotinylated antibody).

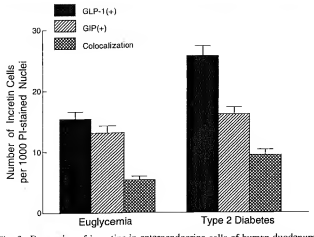

Fig. 3. Expression of incretins in enteroendocrine cells of human duodenum. Cell number is expressed (means ± SE) as total number of cells containing GLP-1 alone, GIP alone, and both colocalized in first and second portions of duodenum, normalized per 1,000 propidium iodide-stained intestinal cell nuclei.

intestine. Human studies from the 1970s were also indicative of glucagon-like immunoreactivity (GLI) in human duodenum in amounts comparable with K cells (7 ± 0.9 for K cells and 1–10 for GLI cells per mm^2) (4, 5).

It is often written in the literature (34, 51) that L cells are found primarily in the distal small intestine and colon. Consequently, indirect neural and/or hormonal "proximal-to-distal" mediators from the upper intestine to distally located L cells have been proposed (43), but their demonstration and potential functional implication in humans have not been established. However, on the basis of our findings from 62 biopsies, by single-cell PCR amplification, and by using multiple antibodies directed against various L cell products, such pathways are indeed nonobligatory. Plasma GLP-1 immediately after a glucose load could arise from duodenal secretion, similarly to GIP, which exhibits a much similar early postprandial secretory pattern in plasma. Because the presence of K cells in the duodenum has never been challenged as straightforward evidence substantiating an obligatory role for duodenal and proximal intestinal origin for the rapid rise of plasma GIP postprandially (although GIP is found in more distal gut portions as well), a similar role should be attributed to the duodenal L cells. Further supporting this conclusion, intestinal resections of jejunum, ileum, or colon did not reduce GLP-1 secretion after an OGTT (46).

Knowledge regarding similar fasting plasma GIP levels and secretion pattern after challenge in subjects with or without T2DM is well established, and our study concurs. Designing a clinical study to prove directly the hypothesis that duodenal L (or K) cells are functionally responsible for the rapid GLP-1 (or GIP) secretion after glucose challenge in man, or to address the relative contribution of the duodenal pool to the total GLP-1 response, would involve invasive techniques with technical and considerable ethical challenges, especially when they involve healthy individuals.

The shape of the incretin cells, open and with long projections toward the lumen, suggests that they are sensing luminal contents. The signaling mechanisms, from the appearance of nutrients in the duodenum to the release of incretins, are unknown. The enzyme glucokinase is the glucose sensor in β-cells (36), where its activity and expression are modulated by a broad array of cell-specific factors, including glucose. It is present in mouse K cells (8), and we have now confirmed its presence in human K and L cells, but its regulation has not been explored in those cells. Glucokinase pathophysiology is an essential feature of T2DM (36), and the ongoing development of glucokinase activators should facilitate investigation of its role in enteroendocrine cells.

It is evident that, in our population of subjects with newly diagnosed T2DM, the bulk of both total and intact GLP-1 is being secreted between 20 and 80 min, reflecting augmented GLP-1 secretion and coinciding with a significant increase in plasma insulin levels between 20 and 60 min, which suggests that the hyper-GLP-1-emia was of physiological relevance (Fig. 1). The increase in plasma GLP-1 levels in T2DM after the glucose load is not likely due to decreased clearance of GLP-1, because clearance of exogenous GLP-1 is similar in nondiabetic and diabetic conditions (17). An additional compensatory role for GLP-1 could be to enhance glucose disposal, because it exhibits insulinomimetic properties in insulin-resistant states (18). Most previous studies (58, 59) have shown that plasma GLP-1 responses after glucose were diminished in T2DM, but in those reports the hyperglycemic state was already known for many years and under pharmacotherapy, and some of the patients might have had gastroparesis.

This late rise in GLP-1 is analogous to the treatment of healthy volunteers with glucosidase inhibitors, which moves carbohydrates into the lower small intestine, causing a late rise in plasma GLP-1 levels (32, 50). In early T2DM, increased gastrointestinal motility might be involved (23), which would also move carbohydrates into the lower bowel. Another possibility, in addition to altered transit time, arises from what is known about the most active glucose transporter (35), GLUT3. In platelets, GLUT3 is localized in α-granules, and, on stimulation of degranulation by thrombin or activation by protein kinase C, GLUT3 transporters get inserted into the plasma membrane, increasing glucose uptake two- to threefold (27). Because we also see GLUT3 in a cytoplasmic location, a similar possibility arises for incretin-containing cells. Because protein kinase C is activated in some cells, for example,

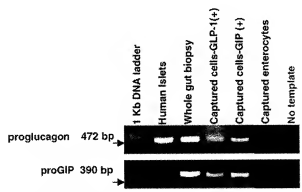

Fig. 4. RT-PCR of laser-captured human duodenal enteroendocrine cells was carried out with specific primer sequences for proglucagon and pro-GIP genes. Both L and K cells (on the basis of specific immunostaining) yield bands at expected PCR product size for both proglucagon and pro-GIP, contrary to captured neighboring enterocytes.

mesangial cells of the kidney, under hyperglycemia (30), glucose transport in L cells may be upregulated. This, in conjunction with the increased number of L cells, could be coupled to increased secretion, but other mechanisms might also be involved (25, 53, 54).

Enteroendocrine cells, like the other three gut cell types (enterocytes, Paneth, and goblet cells), arise from common stem cells in the crypts of Lieberkühn. Their average life span, excluding Paneth cells, is 2–4 days (6). The diabetic milieu may influence the number of stem cell-derived L cells as, in rodents, α-cell numbers and proglucagon expression increase in diabetes (2, 28, 29). The diabetic condition is also clearly responsible for increased plasma glucagon levels seen in T2DM, which again may be tied to the increased number of islet α-cells. Incretin colocalization, especially its apparent modulation by diabetes, strongly suggests that the concept of distinct cell populations dedicated to the synthesis and release of separate peptides should be reviewed under the perspective of a multipotent population of dual incretin (or more peptide)-containing cells predominantly producing GLP-1 or GIP, according to stimuli in the metabolic or biochemical milieu. On the basis of our PCR findings from individually captured cells, it is prudent to assume that these cells contain both messages for proglucagon and pro-GIP and that translation of one or the other (or of both) messages occurs according to yet unidentified homeostatic stimuli, thus exhibiting significant phenotypic plasticity whose regulation warrants further investigation.

Our findings do not refute previous evidence that there is an abundance of L cells and GLP-1 produced in the lower gut; nor do they rule out that secondary nutrient stimulation of the distal gut accounts for most of the circulating GLP-1. Our study objective was to conclusively demonstrate and systematically quantify incretin cells in the human duodenum. Getting distal gut biopsies from healthy volunteers is not an option. However, on the basis of our findings, it would be a surprising paradox that roughly equal numbers of duodenal L and K cells directly exposed to luminal nutrients, with very similar postchallenge incretin plasma concentration curves, could not directly explain the immediate rise of GLP-1 and GIP in plasma. The "proximal-to-distal signal" hypothesis is an alternative theoretical concept that was introduced largely because any data regarding L cells in the duodenum were not definitive in humans, although this has been shown in pig, rat, and dog, suggesting it to also be the case in humans. Similarly to duodenal K cells being responsible for the early phase of postprandial GIP release, duodenal L cells may also be responsible for the early phase of postprandial GLP-1, even though distally located L cells likely play a significant role in later stages of digestion and glucose homeostasis.

On the basis of our findings, we could identify novel cellular targets for pharmacological intervention in metabolic disorders such as obesity and T2DM. Pharmacological concentrations of exogenous GLP-1 (45, 60, 62), GLP-1 analogs resistant to DPP IV (24, 48), and GLP-1 receptor agonists (16) can normalize blood glucose levels and improve early-phase insulin secretion in T2DM, restoring "glucose competence" to β-cells in the process. Understanding the nutrient-sensing mechanisms in enteroendocrine cells might permit us to devise oral secretagogues, perhaps in conjunction with the inhibition of DPP IV (1, 14), whose activity is unaltered at the diabetic state. This should take advantage of the unique benefits of GLP-1 by overcoming clinical concerns about inconvenient routes of administration (38).

In summary, our study provides new insights into the pathophysiology of the human entero-insular axis. The early rise in GLP-1 plasma levels after an OGTT can be linked to the presence, in human duodenum, of L cells to the same extent as K cells.

APPENDIX

Comparison of two assays for GIP-(1–42) and GIP-(3–42)

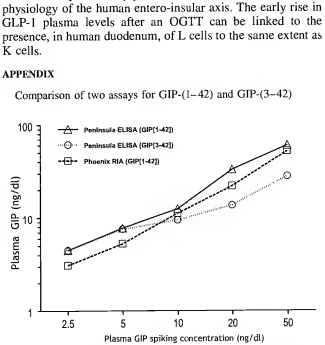

Fig. 5. Fresh (never frozen) plasma, which had been treated with DPP IV inhibitor, was spiked with GIP-(1–42) (Phoenix) in the physiological range and assayed using RIA (Phoenix) and ELISA (Peninsula). An aliquot of the same plasma was also spiked with GIP-(3–42) (New England Peptide, Gardner, MA) at concentrations shown and assayed using ELISA. Results from ELISA and RIA were similar for GIP-(1–42), but the concentration of GIP-(3–42) assayed with ELISA was less accurate than that obtained for GIP-(1–42) in the higher range of concentrations tested. Therefore, ELISA performs better with intact GIP.

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